

**Supporting Online Material for**

**The Molecular Basis for Attractive  
Salt Taste Coding in *Drosophila***

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This PDF includes:

Results

Materials and Methods

Figures S1-7

References

## Supplementary Results

A *Drosophila* DEG/ENaC channel, *ppk11* is proposed to participate in sensing low salt in *Drosophila* larvae (*S1*). However, in adult flies loss of *ppk11* had no impact on the behavioral and electrophysiological responses to low and high salt (fig. S3, A-E). In addition, using a GFP reporter driven by the *ppk11* promoter, *ppk11* was expressed in support cells instead of GRNs in the labellum (fig. S3F), consistent with an independent study (*S2*). Taken together, *ppk11* appeared nonessential for salt sensation in adult animals. Moreover, loss-of-function mutations affecting several known gustatory receptors (GRs) had no impact on the salt responses (fig. S3, A and C).

## Materials and Methods

### Creation of *Ir76b* alleles

To generate deletions in *Ir76b*, we used a standard genetic approach to imprecisely excise a transposable element that inserted in the gene. We obtained a *Drosophila* stock with a P-element that inserted in the third intron of *Ir76b*, (*w*;P{UAST-YFP.*Rab39.S23N*}*Ir76b*<sup>05</sup>; Bloomington Stock Center), and mobilized the transposon by introducing the  $\Delta 2-3$  insertion (*S3*), (*w*; *Sp/CyO*; *ry Sb*<sup>1</sup> P{*ry*[+*t7.2*]= $\Delta 2-3$ }99B/TM6B; Bloomington Stock Center), which provided a source of high levels of transposase. In the F1 generation, several hundred red-eyed male flies were individually crossed to *w*<sup>1118</sup>; TM3,*Sb*/TM6b,*Tb* females. In the F2 generation, white-eyed *Sb*<sup>+</sup> males were selected from each cross and used to perform single-matings with *w*<sup>1118</sup>; TM3,*Sb*/TM6b,*Tb* females. After balanced lines were established, genomic DNA was extracted and the following pair of primers were used to screen for deletions: 5' UTR: 5' TCC ATG ACC CAC TGT CAT GTT TGC ACT TGT 3'; 3' UTR: 5' AGT GCT GTG GTC ACA GGT GTA GAT TGC ACG 3'. After screening several hundred lines, we obtained two deletion alleles. A precise excision line *Ir76b*<sup>R1</sup> was also retained as a control.

### Molecular biology and generation of *Ir76b* transgenic lines

To generate the *Ir76b*<sup>T293A</sup> mutant cDNA, we designed the following pair of primers in which the base pair G:C (marked by stars) substituted for the A:T base pair: forward primer: 5' ACG TCC TTC TAC ACG GCC AAC TTG G\*CC GCC TTC CTG ACC CTT TCC AAG 3'; reverse Primer: 5' CTT GGA AAG GGT CAG GAA GGC\* GGC CAA GTT GGC CGT GTA GAA GGA CGT 3'. We performed the site-directed mutagenesis using the wild-type *Ir76b* cDNA as the template. After verifying the mutation by DNA sequencing, the *Ir76b*<sup>T293A</sup> cDNA was subcloned into the pUAST (S4) vector for germline transformation.

To generate the DNA construct for the *Ir76b*-*GAL4* reporter, a forward (5' GGTTGACCCAGTCTAATGTATGTAATTG 3') and reverse primer (5' CGATACGAGTGCCTACTGTACTCTTTAG 3') were used to amplify a genomic fragment extending ~1.5 Kb upstream of the predicted *Ir76b* transcriptional initiation site. The 1.5 kb promoter region was subcloned into the pCasper4 vector (S5). To generate the *Ir76b*-*QF* construct, the 1.5 kb *Ir76b* promoter region was subcloned into the pattB-QF vector (S6). To obtain the *UAS-Ir76b* and the *UAS-Ir76b*<sup>T293A</sup> constructs, we subcloned the full-length *Ir76b* cDNA clone (IP13656; *Drosophila* Genome Research Center) into the pUAST vector (S4). The transgenic fly lines were generated by germline transformation.

## Fly lines

Existing fly strains used in our studies were: *w*<sup>1118</sup>; *Mi{ET1}ppk11*<sup>MB02012</sup> (Bloomington Stock Center), *QUAS-tdTomato* (Bloomington Stock Center), *Ir25a*<sup>2</sup>

(S7), *Gr33a*<sup>1</sup> (S8), *Gr66a*<sup>ex81</sup> (S8), *Gr64ab*<sup>1</sup> (S9), *UAS-mCD8::GFP* (S10), *Gr5a-l-GFP* (S11), *Gr66a-Gal4* (S12) and *UAS-mCD8-DsRed* (S13).

### **IR76b antibodies**

To produce the IR76b antibodies, we first created a glutathione-*S*-transferase (GST) fusion protein. We PCR amplifying the region encoding the N-terminal 49 amino acids of IR76b using the following primers: 5' AAT AC GAA TTC GCC ACT GGC ATC GAG CTG CTG 3' and 5' ATA AT GCGGCCGC TCA CAG GAC CTC GGT GTA 3', and subcloned the fragment between the EcoRI/NotI sites of the pGEX-6P vector. The purified GST-IR76b-N protein was introduced into a rabbit for antibody production (Covance).

### **Immunocytochemistry**

For immunocytochemical experiments using fly tissues, we dissected probosci and central brains in 0.1 M phosphate buffer (pH 7.2) and fixed the tissue in 4% paraformaldehyde at room temperature for 30 minutes. For performing immunocytochemistry using transfected HEK293T cells placed on coverslips, we washed the cells with 1XPBS buffer, and fixed the cells in 4% paraformaldehyde for 20 minutes. 0.1 M phosphate buffer containing 0.2% Triton X-100 was used for the subsequent primary and secondary antibody reactions. The primary antibodies used were: rabbit anti-IR76b (1:200), rabbit anti-GFP (1:500, Invitrogen), mouse anti-GFP (1:500, Invitrogen), mouse anti-mCD8 (1:10,

Invitrogen) and mouse anti-nc82 (1:50, Developmental Studies Hybridoma Bank). The secondary antibodies (Jackson ImmunoResearch) were: donkey anti-rabbit 488 (1:200), donkey anti-rabbit 549 (1:200), goat anti-mouse 549 (1:200) and goat anti-mouse 488 (1:200). The images were acquired using a confocal microscope. Imaris software was used to reconstruct three-dimensional confocal Z stacks.

### **Behavioral assays**

To conduct the two-way choice assays, we developed a simple test using a Petri dish (10 mm × 35 mm). We separated each Petri dish into two zones, using acrylic plexiglass sheets (3 mm in width, 3 mm in height and 35 mm in length), which we melted to the bottom of each dish. We added the tastants plus either red (sulforhodamine B, 0.02% final concentration) or blue (brilliant blue FCF, 0.01% final concentration) dye to 1.0% agar and loaded the mixture to each half of the dish. ~70 two-day old adult flies were used per assay. The animals were starved for 24 hours before the two-way food choice assays. We did not use CO<sub>2</sub> anesthesia to transfer the flies to the Petri dishes as CO<sub>2</sub> might have an impact on the responses. Rather, we immobilized the flies by inserting the fly vials into a water-ice mixture for two minutes, and then gently tapped the flies into the Petri dishes. Flies were allowed to feed in a Petri dish for 90 minutes and the numbers of flies with blue (N<sub>B</sub>), red (N<sub>R</sub>) or purple (N<sub>P</sub>) abdomens were scored in blind fashion using light microscopy. Preference indexes (PIs) were calculated

according to the following formulas:  $PI = (N_B + \frac{1}{2} N_P) / (N_B + N_R + N_P)$  or  $(N_R + \frac{1}{2} N_P) / (N_B + N_R + N_P)$ .

### **Assaying tastant-induced action potentials using tip recordings**

Tip recordings were performed as we described previously using labellar sensilla (*S14*). Briefly, 1 – 2 day old adult animals were used for the physiological assays. For all the tastant solutions tested, including NaCl, sucrose and water, we used 1 mM KCl as the electrolyte in the recording electrode. The reference electrode contained Ringer's solution (140 mM NaCl, 3 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM D-glucose, 10 mM HEPES, pH 7.4).

### **Whole-cell recordings of IR76b-expressing HEK293T cells**

To express IR76b *in vitro*, we subcloned the full-length *Ir76b* or *Ir76b*<sup>T293A</sup> mutant cDNA into the pcDNA3 vector to create pcDNA3-*Ir76b*. The pcDNA3-*Ir76b* or pcDNA3-*Ir76b*<sup>T293A</sup> construct was co-transfected along with pcDNA3-*GFP* (1:10 ratio) into HEK293T cells. After 24-48 hours, live GFP positive HEK293T cells were identified using fluorescent microscopy. Cells that were GFP negative were used as controls.

To perform whole-cell voltage clamp recordings, we prepared glass pipets using borosilicate glass (OD. 1.5 mm). The electrodes had a resistance of 2-5 MΩ after fire polishing, and were filled with a solution containing 150 mM CsMes, 2 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4. To selectively measure 150 mM Na<sup>+</sup>

conductance, we used an isotonic solution with  $\text{Na}^+$  as the only cation species. (150 mM NaCl, 10 mM D-glucose and 10 mM HEPES, pH 7.4). When decreasing the  $\text{Na}^+$  concentrations from 150 mM to 50 mM, 100 mM NMDG<sup>+</sup> was added to keep the solution isotonic. As a control, the  $\text{Cl}^-$  was replaced with the same concentration of gluconate anions (150 mM sodium gluconate, 10 mM D-glucose and 10 mM HEPES, pH 7.4). To measure a possible  $\text{K}^+$  conductance, we used an isotonic solution with  $\text{K}^+$  as the only cation species (150 mM KCl, 10 mM D-glucose and 10 mM HEPES, pH 7.4). To measure the relative cation permeability to  $\text{Cs}^+$ , we used the equation:  $P_{X^+}/P_{\text{Cs}^+} = [\text{Cs}^+]_i^{\exp(E_{\text{rev}}F/RT)}/[X^+]_o$ .  $[X^+]_o$  represents  $\text{Na}^+$  or  $\text{K}^+$  concentration outside of the cells.  $[\text{Cs}^+]_i$  represents the internal  $\text{Cs}^+$  concentration in the glass pipette. Abbreviations:  $E_{\text{rev}}$ , reversal potential;  $F$ , Faraday constant;  $R$ , gas constant;  $T$ , absolute temperature.

Whole-cell patches were achieved after gently breaking into the plasma membrane with the tips of the electrodes. We used the Clampex program, and applied a series of 500 ms voltage pulses ranging from -100 mV to +100 mV in 10 mV increments to the cells with a holding potential of 0 mV. The current signals were processed with a patch clamp amplifier and analyzed using Clampfit software.

### **Sequence Alignment and Homology Modeling**

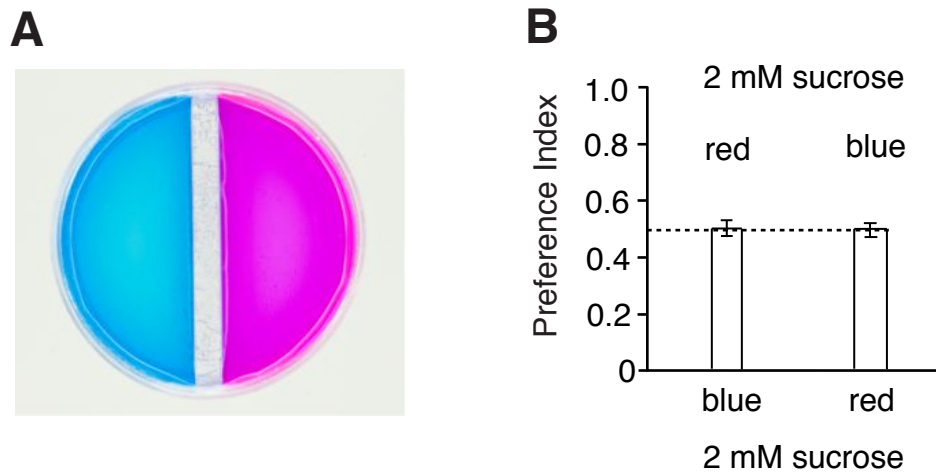
We used ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) to perform multiple-sequence alignments for the iGluR family members. To model the three-dimensional structure of IR76b using the crystal structure of rat GluA2

(PDB code: 3KG2), we used software available at the Swiss-Model server (<http://swissmodel.expasy.org/>).

### **Statistical analysis**

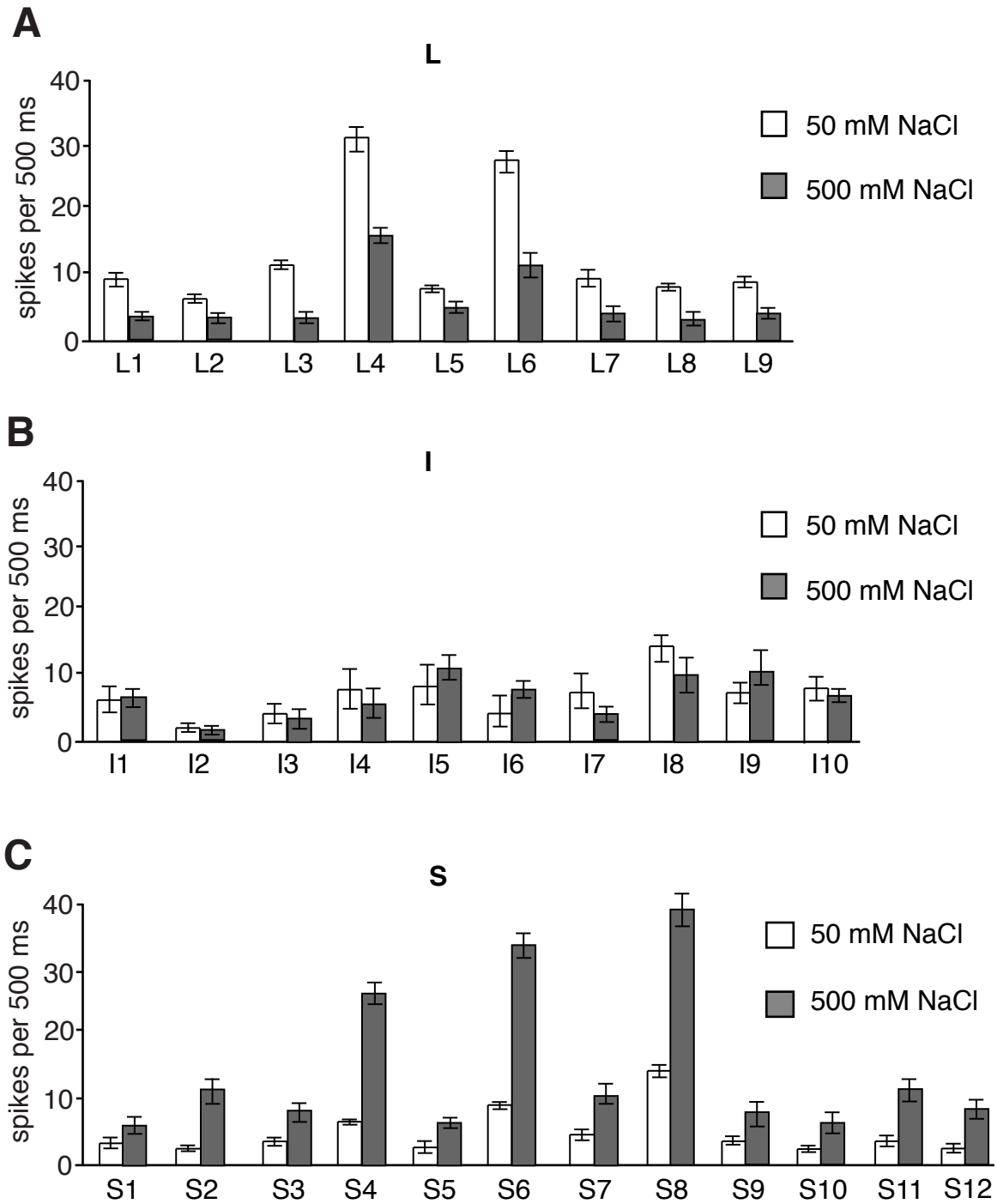
We analyzed the tip recording data using either unpaired Student t-tests for comparing two sets of data, or ANOVA tests for comparing multiple samples. We used non-parametric Mann-Whitney tests and Siegel-Tukey tests for determining the statistical significances of pairwise comparisons, and the Kruskal-Wallis test for comparing multiple comparisons.

# Fig. S1

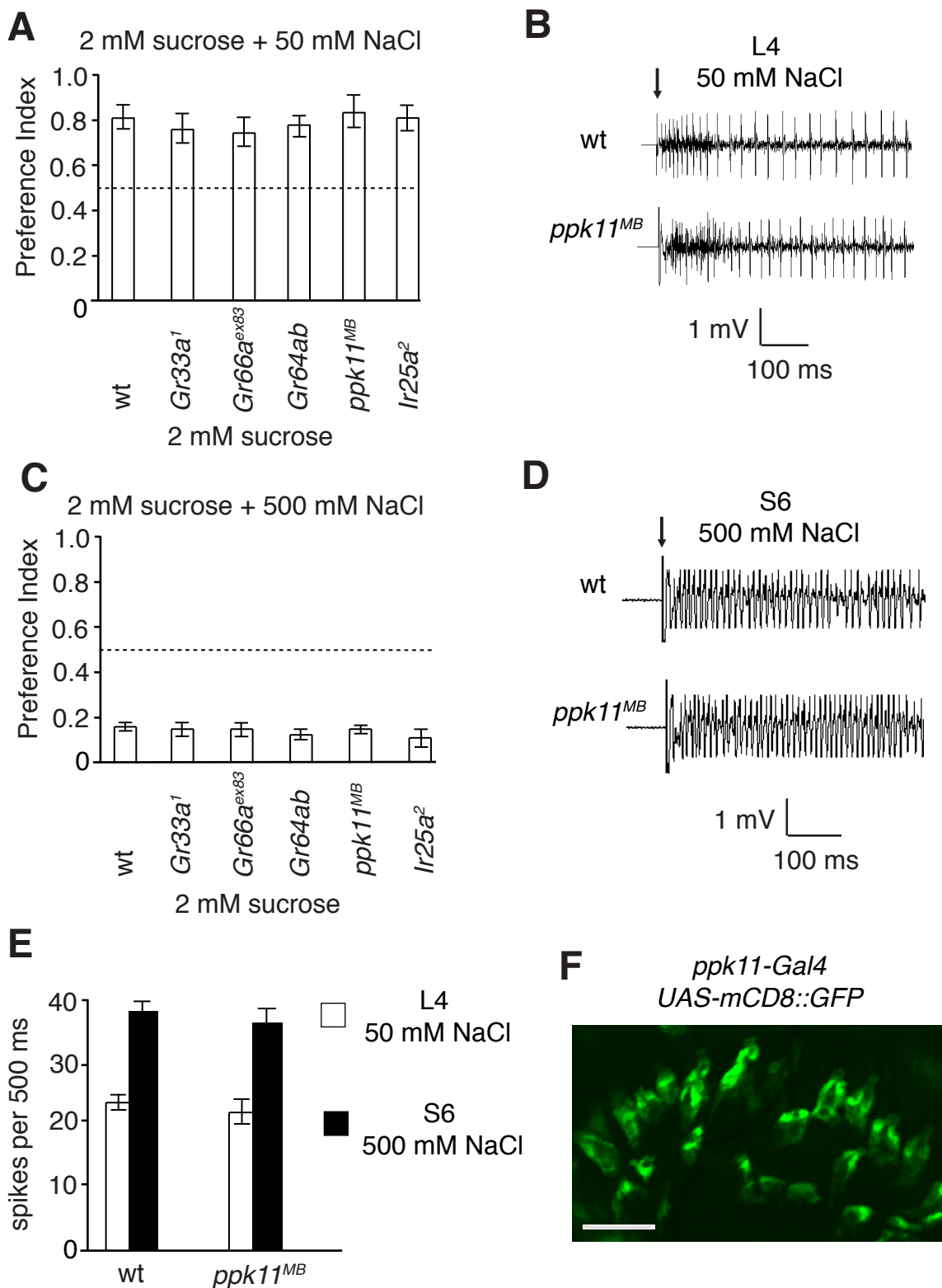


**Fig. S1. Two-way choice assays.** (A) Petri dish set-up used in the two-way gustatory choice assays (each trial included ~70 flies). (B) Lack of effects of red and blue food-coloring on taste discrimination using two way choice tests (2 mM sucrose on both sides). n = 10.

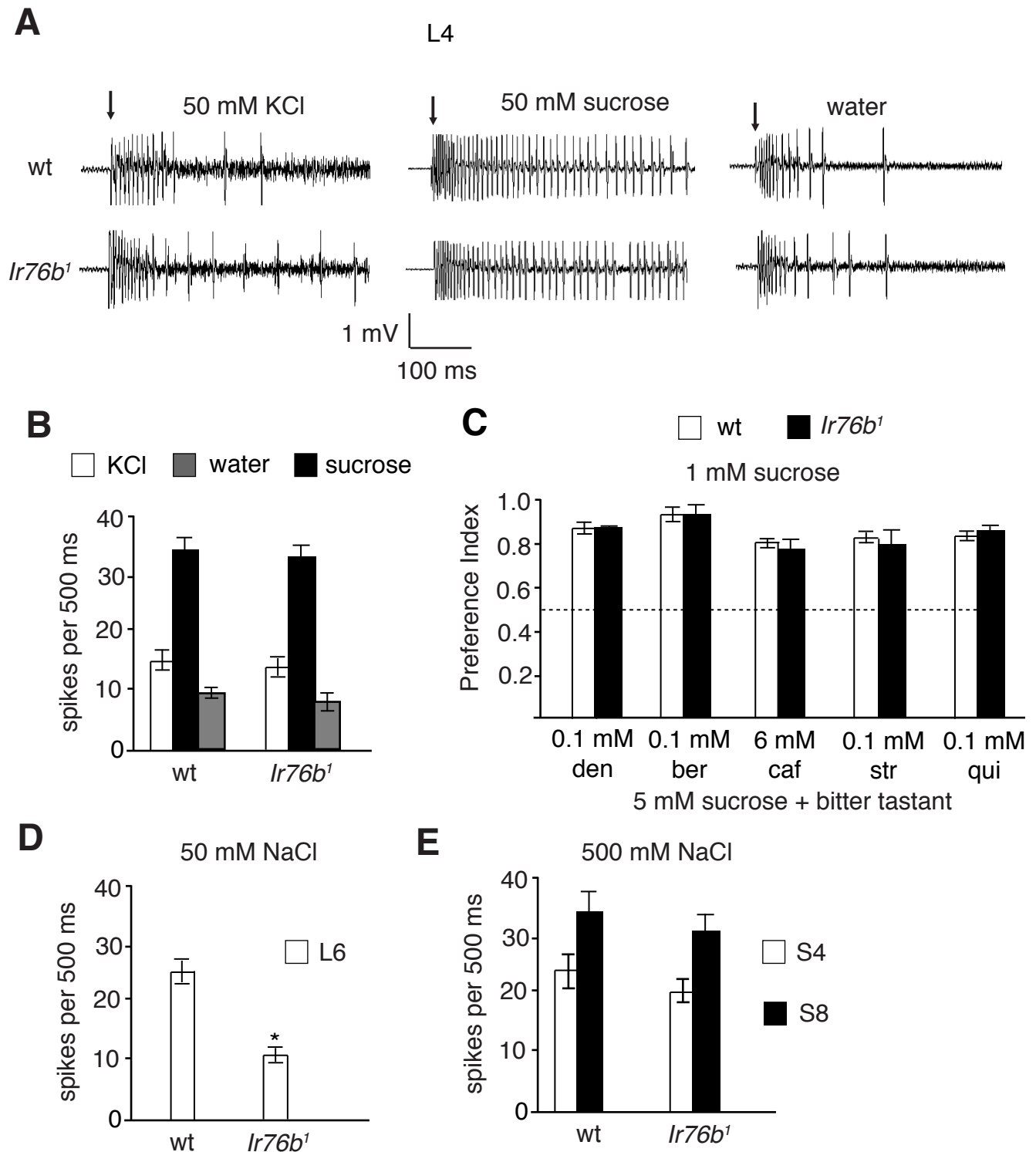
# Fig. S2



**Fig. S2. Firing frequencies of L-type, I-type or S-type sensilla in response to low and high salts. (A)** The firing frequencies among nine L-type sensilla in response to low salt (50 mM NaCl) and high salt (500 mM NaCl). **(B)** The firing frequencies among 10 I-type sensilla in response to low salt (50 mM NaCl) and high salt (500 mM NaCl). **(C)** The firing frequencies among 12 S-type sensilla in response to low salt (50 mM NaCl) and high salt (500 mM NaCl).  $n > 3$ .

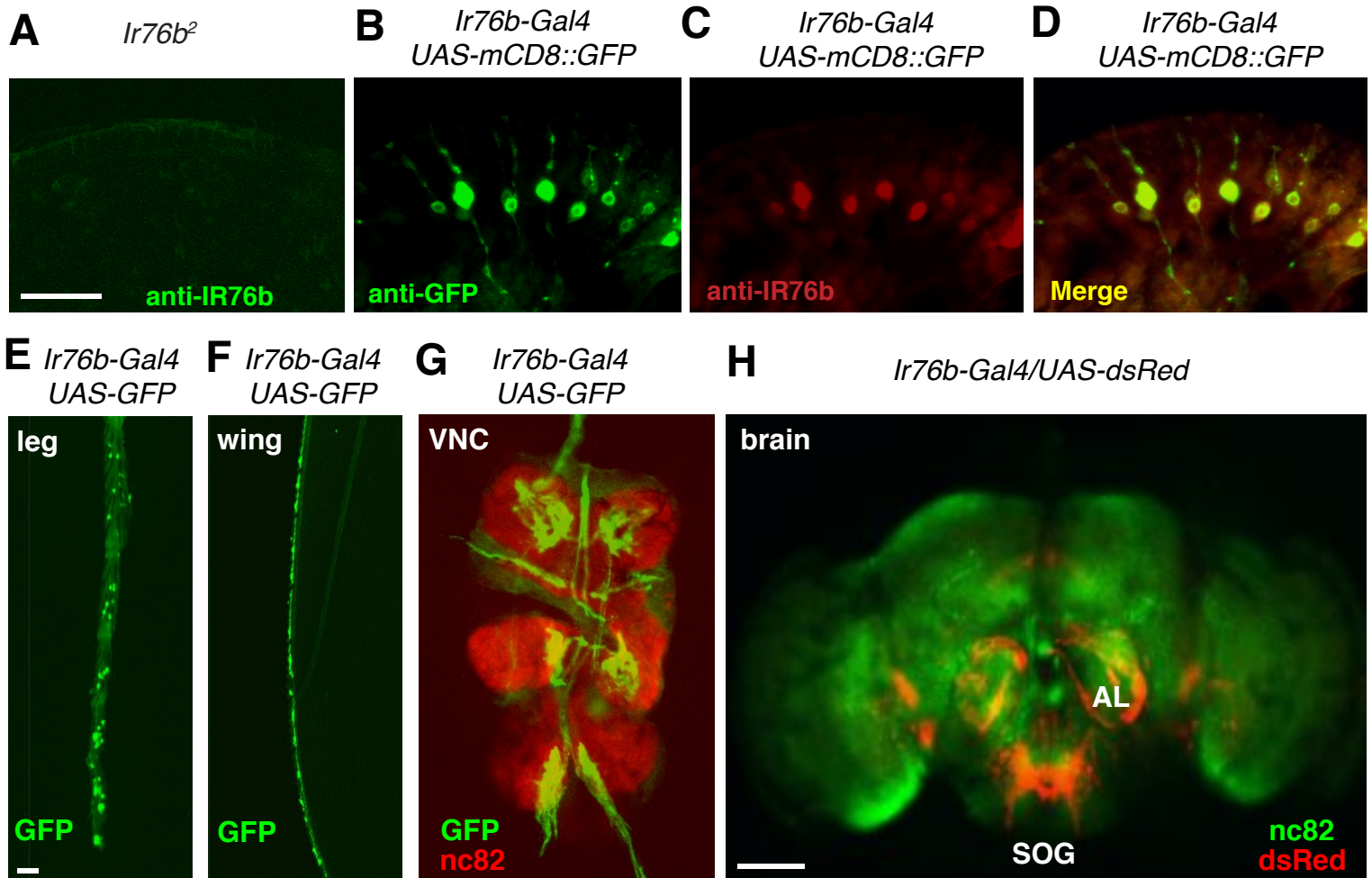


**Fig. S3. The responses to low salt (50 mM NaCl) and high salt (500 mM NaCl) among wild-type (wt) flies and animals with mutations affecting GRs, IR25a and Ppk11. (A)** Behavioral responses using two-way choice tests (2 mM sucrose versus 2 mM sucrose plus 50 mM NaCl).  $n = 3$ . **(B)** Tip recordings using L4 sensilla of the indicated genotypes in response to 50 mM NaCl.  $n = 5$ . **(C)** Behavioral responses using two-way choice tests (2 mM sucrose versus 2 mM sucrose plus 500 mM NaCl).  $n = 3$ . **(D)** Tip recordings showing the firing frequencies of GRNS in S6 sensilla in response to 500 mM NaCl. **(E)** Statistical analysis of the number of action potentials elicited by 50 mM and 500 mM NaCl, respectively in wild type and *ppk11<sup>MB</sup>*.  $n = 5$ . **(F)** The expression pattern of *ppk11* in the proboscis. The scale bar indi-



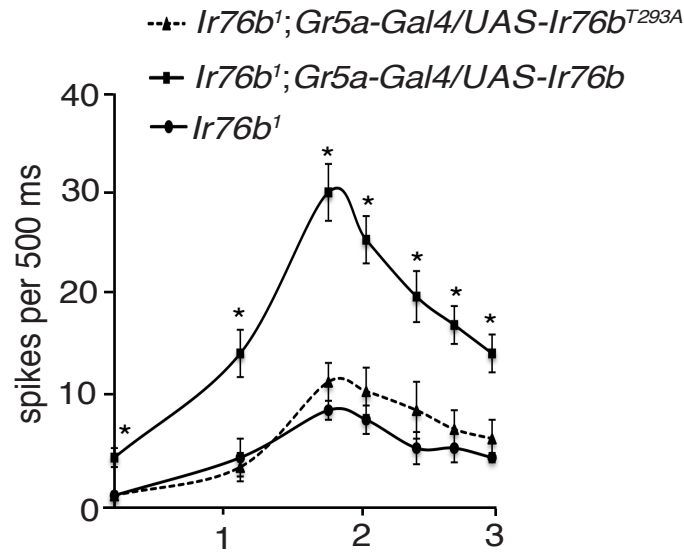
**Fig. S4. Physiological and behavioral responses to KCl, sucrose, water and bitter tastants in wild type and *Ir76b* mutant animals.** (A and B) Sample tip recordings and quantifications of action potentials elicited by L4 sensilla in response to 50 mM KCl, 50 mM sucrose or water in wild type and *Ir76b* mutant animals. *n* = 5. (C) Behavioral responses to bitter tastants in wild type and *Ir76b* mutant animals (1 mM sucrose versus 5 mM sucrose plus the indicated bitter tastants). The bitter chemicals tested were: 0.1 mM denatonium (den), 0.1 mM berberine (ber), 6 mM caffeine (caf), 0.1 mM strychnine (str) and 0.1 mM quinine (qui). *n* = 3. (D) Firing frequencies of GRNs in L6 sensilla in response to 50 mM NaCl in wild type and *Ir76b* mutant animals. *n* = 5. (E) Firing frequencies of GRNs in S4 or S8 sensilla in response to 500 mM NaCl in wild type and *Ir76b* mutant animals. *n* = 5. \**p* < 0.01. Unpaired

# Fig. S5

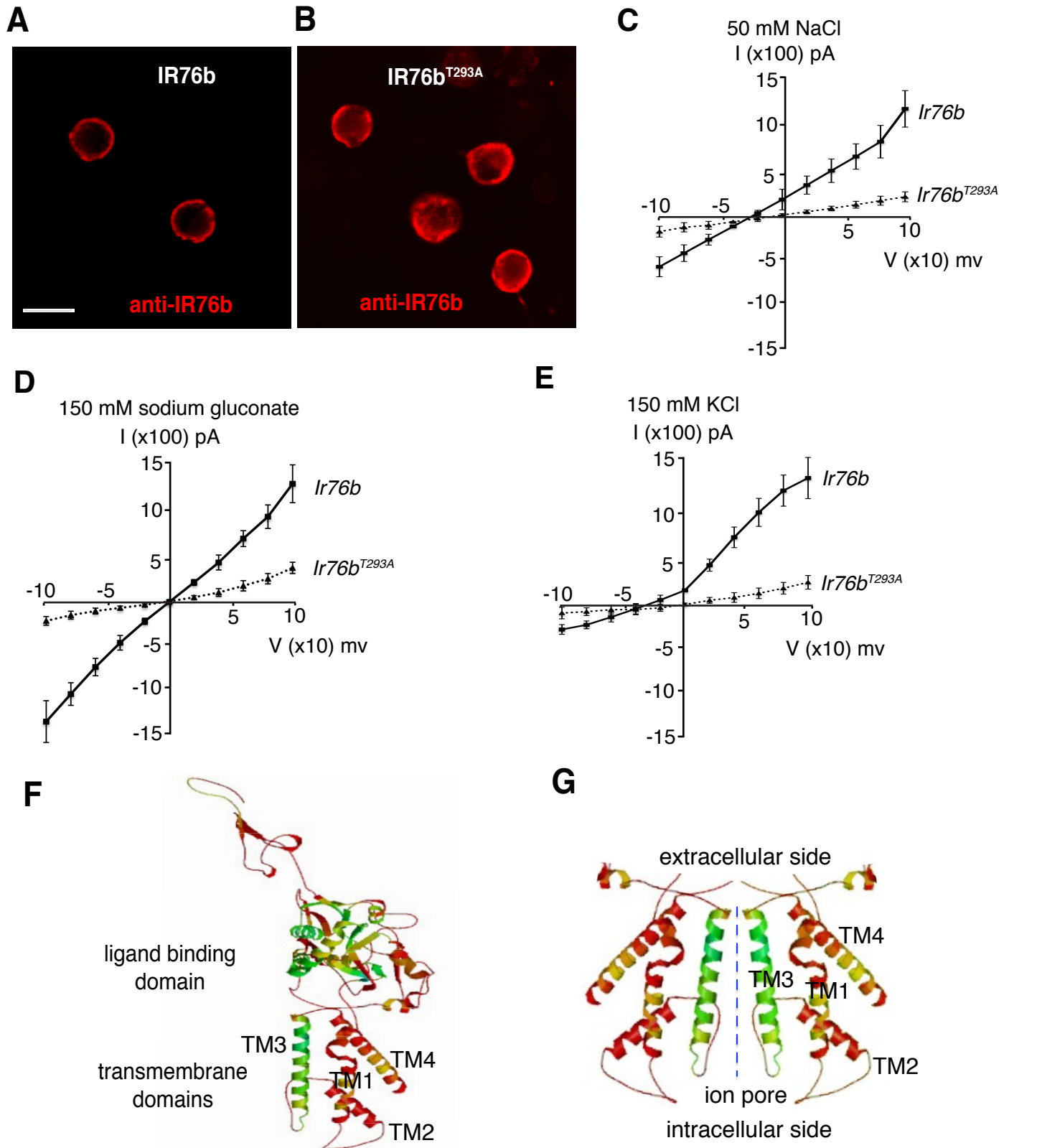


**Fig. S5. Anti-IR76b and anti-GFP co-staining in *Ir76b-Gal4/UAS-mCD8::GFP* animals, and central brain projection patterns of *Ir76b*-expressing GRNs.** (A) Immunostaining of labelle in *Ir76b*<sup>b2</sup> mutant animals. (B-D) Double labeling of the labelle of *Ir76b-Gal4/UAS-mCD8::GFP* flies with anti-GFP and anti-IR76b. (E) Immunostaining of the tarsi from forelegs in *Ir76b-Gal4/UAS-mCD8::GFP* animals with anti-GFP. (F) Immunostaining of the wing margins in *Ir76b-Gal4/UAS-mCD8::GFP* animals with anti-GFP. (G) Immunostaining of the ventral nerve cord (VNC) in *Ir76b-Gal4/UAS-mCD8::GFP* animals with anti-GFP and anti-nc82, which marks the neuropiles. (H) Immunostaining of the entire brain of *Ir76b-Gal4/UAS-mCD8::dsRed* adult flies with anti-dsRed and anti-nc82, which marks the central brain. Axons of *Ir76b* expressing chemosensory neurons in the proboscis and the antenna, respectively innervated the SOG and the antennal lobe (AL) in the central brain. The scale bar indicates 10  $\mu$ m.

# Fig. S6



**Fig. S6. Tip recordings and behavioral assays after ectopically expressing *Ir76b* or *Ir76b*<sup>T293A</sup>.** Action potentials elicited by L2 sensilla in response to the indicated concentrations of NaCl after misexpression of *Ir76b* (*UAS-Ir76b*) or *Ir76b*<sup>T293A</sup> (*UAS-Ir76b*<sup>T293A</sup>) in sugar responsive *Gr5a* GRNs under control of the *Gr5a-Gal4*.  $n > 3$ . \* $p < 0.05$ . ANOVA tests.



**Fig. S7. Patch-clamp analysis of the ion permeability of IR76b or IR76b<sup>T293A</sup> expressed in HEK293T cells.** (A and B) Immunostaining of HEK293T cells co-transfected with vectors expressing *Ir76b* or *Ir76b<sup>T293A</sup>* using anti-IR76b. (C-E) I-V relationships of HEK293T cells expressing *Ir76b* or *Ir76b<sup>T293A</sup>*. Voltage ramps were applied from -100 mV to +100 mV to the cells with increments of 10 mV. The external saline contained the indicated salts. (C) 50 mM NaCl. The bath included 100 mM NMDG<sup>+</sup> to keep the saline isotonic. (D) 150 mM sodium gluconate. (E) 150 mM KCl. (F and G) Homology modeling of the three-dimensional structure of IR76b using the structure of rat GluA2 (S15) as the template. The dashed line in G indicates the midline of the ion pore of IR76b. TM1-4 represent the four transmembrane segments. The scale bar indicates 10  $\mu$ m.

## Supplemental References

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